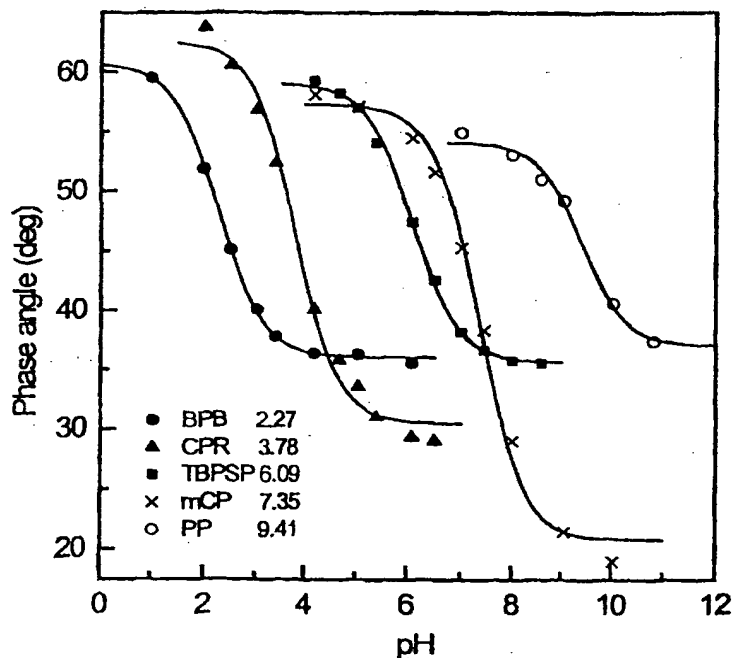




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(54) Title: ASSAY METHOD AND DEVICE



(57) Abstract

A FRET assay uses fluorescent metal ligand complexes as donor molecules and assays for pH or for other analytes, the presence of which can change the pH in the environment of an acceptor molecule.

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## Assay Method And Device

### Description

#### Field of the Invention

This invention relates generally to the field of assays and more particularly to fluorescence intensity assays.

#### Background

Optical measurements of pH are of wide interest in analytical and clinical chemistry. At present, optical sensing of pH is based on measurements of steady-state fluorescence intensity, as determined by the absorptive and emission properties of a sample. Time-resolved fluorescence has also been demonstrated for pH sensing using a number of lifetime pH sensitive probes. However, most lifetime-pH sensitive probes display relatively short lifetime in the range of several nanoseconds which makes their use difficult in practical inexpensive pH sensing. Though there are several reports about long lifetime luminescent inorganic complexes that show pH-sensitive luminescence characteristics, these Ru(II) based pH-sensitive complexes show pH-dependent emissions, mostly in the low pH range from 0 to 6. Also, there is limited work on embedding metal ligand complex (MCL) pH probes into a polymeric support where a shift in their pH sensitivity relative to that in liquid solution is expected. The low pH range limits the sensors to be used for biomedical purposes, where a sensor capable of monitoring pH value around 7.5 is often required.

An alternative approach to lifetime-based pH sensing uses fluorescence energy transfer (FRET) for pH sensing. In this case the sensors contain a fluorescence donor and a pH-sensitive acceptor. The fluorescence donor can be selected for its absorption, emission, quantum yield, and decay time characteristics without concern for its sensitivity to pH. The acceptor can be fluorescent or non fluorescent, but must display absorption spectral change in response to pH in the wavelength range of the donor emission, and be photostable. The donor and acceptor molecules can be covalently linked or simply mixed together. With the FRET approach, sensors with different pH-

sensitive range can be readily developed by simply selecting an acceptor with an appropriate pKa value from the vast pool of inexpensive and commercially available colorimetric indicator dyes.

The rate of the FRET depends on the integral overlap of the donor emission with the acceptor pH-dependent absorption, the distance between donor and acceptor and the relative orientation of the donor and acceptor transition dipoles. Some fluorescence lifetime features of covalently linked and mixed donor-acceptor systems for pH sensing has been already simulated. Recent reports of using Ru(II) complexes as an energy donor were concerned with sensing of glucose and in immunoassays. Metal-ligand probes display attractive spectral properties: are excitable with LED and diode lasers, exhibit large Stokes shift (~200 nm), and have acceptable quantum yields. In addition, the most attractive property of MLC probes is long luminescence lifetime in the order of several hundreds nanosecond to microseconds. The long lifetime allows of using low modulation frequency in phase-modulation technique. The low modulation frequency in the range of several hundred kHz is desirable in terms of the cost of practical device for sensing.

In addition, the long lifetime of donor allows for significant molecular diffusion of donor and acceptor molecules which enhances the FRET. This may results in using lower acceptor concentrations than calculated based on Förster theory. Therefore, the FRET-based pH sensors with MLC as donors may require lower acceptor concentrations compared to that with a nanosecond lifetime donors. It is known that for sufficient energy transfer (more than 0.70), the acceptor concentrations need to be in the range of 3 -50 mM for donor-acceptor system with a Förster distance  $R_0$  from about 50 to 20 Å, respectively. Such acceptor concentrations results in high optical densities at the excitation and emission wavelengths making intensity measurements difficult to use in a quantitative manner. At these high acceptor concentrations there are, in addition to FRET, a significant inner filter effect which depends on the effective optical path lengths. The changes in the effective path lengths are not easy controlled which were a major problems in development of such pH sensors using an intensity based methods. Lifetime-based technique does not depend on optical density of the sensors which allow easy design of pH FRET-based sensors.

There is a commercial need for a sensor mechanism that can be adapted to sense a variety

of analytes. It is an object of this invention to provide such a sensor mechanism that can be used in an assay.

#### Summary of the Invention

The present invention provides a FRET arrangement for sensing the concentration of protons supported by the acceptor. Energy is generated by subjecting a donor molecule comprising a fluorescent MLC to exciting radiation. The resulting energy will result in detectable fluorescence that may be diminished if acceptor molecules in the vicinity of the energy-producing MLC have a modified hydrogen ion status sufficient to cause them to accept more energy. Changes in the fluorescence produced are detected and correlated with the presence or amount of the analyte responsible for the change in the pH or hydrogen ion environment of the acceptor.

MLC's such as Ru(II) complexes are useful as a donors and pH-sensitive indicators such as phthaleins are useful as acceptors. The donors and acceptors may be held in polymeric supports. The assays and devices of the present invention are useful for detecting a pH range from about 1 to about 11 and can be measured using a single fluorescence donor and different pH-sensitive acceptors which possess different pKa's. pH can be sensed directly by the present assay and device. Also, the device and method of this invention can be used to indirectly sense the presence or amount of other analytes including CO<sub>2</sub>, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Cl<sup>-</sup>, ammonia, urea and others that induce the changes in hydrogen ions in the sensing element. Induced changes in H<sup>+</sup> within sensing element can be transduced via energy transfer to measure an analyte concentration in the sample. This can be usually achieved by using a proper polymers which are designed for specific analytes.

#### Brief Description of the Drawings

FIG. 1 shows absorption spectra of pH indicator Cholophenol Red in pH 4.16 (.....) and pH 8.35 (- - -) and absorption and emission spectra of [Ru((dpp)(SO<sub>3</sub>Na<sub>2</sub>)<sub>3</sub>) Cl<sub>2</sub>] as a donor (-----) in EC films. The grey area indicates the spectral overlap.

FIG. 2 shows Intensity decays of  $[\text{Ru}(\text{dpp})(\text{SO}_3\text{Na}_2)_3]^{2+}$  in the presence of pH indicator. CPR at its several concentrations in EC films at pH 8.35. Intensity decays are represented as a frequency-dependent phase angles and modulations.

FIG. 3 shows pH-dependent intensity decays of  $[\text{Ru}(\text{dpp})(\text{SO}_3\text{Na}_2)_3]^{2+}$  in the presence of 8.8 mM of CPR in EC films. Intensity decays are represented as a frequency-dependent phase angles and modulations.

FIG 4 shows pH calibration curves for FRET-based sensor  $[\text{Ru}(\text{dpp})(\text{SO}_3\text{Na}_2)_3]^{2+}$ -CPR using time-resolved data, phase angles and modulations at frequency of 363 kHz.

FIG 5 shows pH calibration curve for FRET-based sensor with two pH indicators as acceptors  $[\text{Ru}(\text{dpp})(\text{SO}_3\text{Na}_2)_3]^{2+}$ -(CPR+TBPSP). Arrows indicate the ranges where CPR and TBPSP are sensitive to the pH.

FIG 7 shows Effect of polymeric support on performance of  $[\text{Ru}(\text{dpp})(\text{SO}_3\text{Na}_2)_3]^{2+}$ -TBPSP FRET pH sensor. PH-dependent phase angles were measured at frequency of 300 kHz. Dashed line shows the pH dependent absorbance of TBPSP in buffer.

### Detailed Description

Materials used:

Tris(disodium 4,7-diphenyl-1,10-phenanthroline disulfonate)ruthenium(II) chloride  $(\text{Ru}(\text{dpp})(\text{SO}_3\text{Na}_2)_3 \text{Cl}_2)$  was used as a donor. The synthesis and spectral characteristics of  $(\text{Ru}(\text{dpp})(\text{SO}_3\text{Na}_2)_3 \text{Cl}_2)$  has been recently described. The FRET acceptors were chosen from the pH-sensitive phthaleins like m-cresol purple (mCP), bromothymol blue (BTB), chlorophenol red (CPR), 3,4,5,6-tetrabromophenolsulfophthalein (TBPSP), and phenolphthalein (PP) to cover wide range of pH values from 1 to 10 were purchased from Aldrich (Milwaukee, WI). The pH indicators were used without further purification. Chemicals, ethyl cellulose (EC), tetraethyl orthosilicate (TEOS), poly(2-hydroxypropyl methacrylate (PHPMA), tributyl phosphate (TBP), tetraoctylammonium bromide (TOABr), methanol, ethanol, and toluene, were also from Aldrich.

The sensors characterization were performed in phosphate buffer with 140 mM NaCl, air equilibrated at room temperature of 23 °C.

### *Sensor Film Preparation*

For EC-, PVC-, PVB- and PHPMA-based sensors, the procedures involved first adding 34.2 µl of 10 mM solution of  $\text{Ru}[(\text{dpp})(\text{SO}_3\text{Na}_2)]_3\text{Cl}_2$  in methanol and 10 µl of TBP into 0.1 ml of either EC, PVC, PVB solution (33.2 mg in 0.8 ml of toluene and 0.2 ml of ethanol), or PHPMA solution (100 mg in 1 ml of methanol). Then, added into the above solution were the solution of a given acceptor in methanol, 0.5 M solution of TOABr in methanol, and methanol in such quantities that 171 µl of the final coating solution contained 70 mM of TOABr. Sensor films were made by applying 4-5 µl of the given coating solution onto the surface of glass support, and let it be dried at ambient atmosphere for about 10 minutes.

For sol-gel based sensor, the procedure was adapted as described in [27] with slight modification. First, 40 µl of 10 mM donor solution was mixed with 18 µl of 8.5 mM TBPSP solution and 20.7 µl of 0.5 M TOABr solution in a 4 ml glass vial. The mixture was allowed to dry at room temperature. Next, added into the glass vial were 150 µl of TEOS, 25.8 µl of de-ionized water, 17.2 µl of 0.04 M hydrochloric acid and 7 µl of methanol. The precursor solution was then sonicated at room temperature for 2 h, and kept at about 4° C for 12 h. The sensor film was then made from the solution by dip-coating.

### *Frequency-domain measurements*

Multi-frequency phase and modulation data were collected on the frequency-domain instrumentation ISS K2 (ISS Inc., Urbana Champagne, IL). For the excitation we used blue LED (Nichia NSPB500S) with maximum at 457 nm which was intensity modulated with driving current. This light source was chosen because it is practical and inexpensive for analytical or clinical sensing.

Emission light was collected through a long pass filter above 595 nm. The measurements were carried out at room temperature of 22° C.

In phase modulation fluorometry, the sample is excited with an intensity-modulated light and the fluorescence is emitted with the same modulation frequency. The experimental observables are the phase shift of the emission ( $\theta$ ) and its modulation ( $m$ ), both relative to the phase and modulation of the excitation light source [28]. These quantities may change from 0 to 90 degree and from 1 to 0 and are related to an apparent phase ( $\tau_p$ ) and modulation ( $\tau_m$ ) lifetimes by

by

$$\theta = \text{atan}(\omega\tau_p), \quad m = (1 + \omega^2 \tau_m^2)^{-1/2}, \quad (1)$$

where  $\omega$  is the circular modulation frequency ( $\omega = 2\pi f$ ). Phase and modulation of the donor emission reflects the changes in an intensity decay due to FRET from donor to the pH-sensitive acceptor. A functional sensor for pH does not require measurement of a complete frequency response. Measurement at single-modulation frequency is adequate for quantitative pH sensing [20].

## Results

### *Absorption and Emission Spectra*

Fig.1 shows the representative absorption and emission spectra of  $\text{Ru}[(\text{dpp})(\text{SO}_3\text{Na}_2)]_3 \text{Cl}_2$  (Donor) and pH indicator, CPR (Acceptor) in EC films. One of the most important variables of D-A system is the degree of spectral overlap between emission spectrum of the donor and absorption spectrum of acceptor. The importance of integrated spectral overlap of pH sensor can be understood by examining Fig.1. Protonation of CPR shifts its absorption maximum from 592 nm (large spectral overlap, indicated by grey area) to 405 nm (very small spectral overlap, not shown). Therefore, as the pH value decreases, the energy transfer efficiency from donor to acceptor will also decrease. In



consequence the intensity and the lifetime of the donor should be pH dependent. Similar integrated spectral overlaps were determined for other donor - acceptor systems (Table I). The second important variable of D-A system is the distance between D and A which allows for FRET to occur. FRET occurs if the D and A distance is comparable to the critical Förster distance,  $R_0$ , and not be larger than about  $2R_0$  [24]. In this report we have mixed donor and acceptor molecules in liquid solution and then formed the solid polymers. We anticipated that acceptor molecules are uniformly and randomly distributed around the excited donor molecules. In such case the steady-state intensity and the intensity decay of the donor emission can be described as a function of acceptor concentration [24]. Valuable information about the acceptor concentration needed is the value of critical acceptor concentration,  $C_0$ , which can be calculated from the value of  $R_0$ . In Table I are summarized calculated values of  $R_0$  for several donor-acceptor systems. It should be noted that acceptor concentration equal to  $C_0$  corresponds to the energy transfer efficiency of 0.72 (three-dimension solution). The  $R_0$  and  $C_0$  values are estimated values and may be slightly different in various polymeric supports. The accurate value of  $R_0$  is difficult to determine because the values including quantum yield of donor, value of refractive index, efficiency of oxygen quenching are specific for each polymeric support. Following assumptions have been applied to calculate the  $R_0$  values : (1) molar extinction coefficients of pH indicators were determined in aqueous buffer solutions, (2) quantum yield of donor of 0.32 (in water in the absence of oxygen), (3) refractive index of 1.33, and (4) dipole orientation factor of 2/3 because of long lifetime of the donor. Nevertheless, these values are valuable information for design and development of pH-FRET based sensors.

For polymeric matrices it is somewhat difficult to estimate the acceptor concentration because of several factors such as reduced volume during drying of the polymer, expected micro heterogeneity of polymers, and possible molecular diffusion. For optimal pH sensor performance the acceptor concentration need to be determined experimentally. Using time-resolved data the experimental values for  $R_0$  can also be determined and compared with those calculated from spectral characteristics of donor

and acceptor molecules.

### *Intensity Decays*

Figure 2 illustrates intensity decays (phase angles) of  $[\text{Ru}(\text{dpp}(\text{SO}_3\text{Na}_2)_3)]$  in the presence of various acceptor concentrations (CPR) in EC films at pH 8.35 (base form of CPR). The acceptor concentrations in the EC films were estimated to be approximately 8-fold higher than that in the coating solution due to the reduced volume of EC after drying. It has been found that the experimental values of  $R_0$  for  $[\text{Ru}(\text{dpp}(\text{SO}_3\text{Na}_2)_3)]$ -CPR in EC were comparable to that expected from spectral overlap (Table 1). This agreement indicates that the donor and acceptor molecules are distributed randomly and there is no translational motion of molecules. The extent of energy transfer from  $[\text{Ru}(\text{dpp}(\text{SO}_3\text{Na}_2)_3)] \text{Cl}_2$  to CPR increases with the increase of CPR concentration, which is indicated with decreased lifetime (frequency-responses are shifted towards higher frequencies). Examination of the acceptor concentration effect on energy transfer efficiency provides an information of required concentration for sufficient FRET to obtain substantial changes in time-resolved parameters such as phase angle and modulation. In the case presented in Figure 2 the FRET efficiency is consistent with increased CPR concentration as 0.63 (2.4 mM), 0.86 (5.6 mM), and 0.93 (8.8 mM).

Figure 3 shows pH dependent intensity decay of  $[\text{Ru}(\text{dpp}(\text{SO}_3\text{Na}_2)_3)]$  - CPR in the pH range from 4.16 to 8.35. The estimated concentration of acceptor in EC film was 8.8 mM. It is evident that with increased pH value from 4.16 to 8.35, the FRET also increased, resulting in shorter lifetimes. The increase of FRET is due to increased concentration of the base form of CPR at higher pH values. Phase angles and modulation dependencies on pH, which are shown in Figure 3, allow choosing a modulation frequency at which there are substantial pH sensitivity on phase and modulation. For  $[\text{Ru}(\text{dpp}(\text{SO}_3\text{Na}_2)_3)]$ -CPR in EC film one may choose a modulation frequency from 100 kHz to 1 MHz.

#### *pH Sensing With Phase and Modulation*

There is a wide range of modulation frequencies at which there are observed significant differences in phase and modulation between  $D + A_{\text{acid}}$  (pH 4.16) and  $D + A_{\text{base}}$  (pH 8.35, see Figure 3). Larger difference means higher phase and modulation sensitivity to pH changes. pH calibration curves were plotted using phase angles and modulations at frequency of 363 kHz. There are substantial magnitudes of changes in phase and modulation of about 49 deg and 52 %, respectively. Such large changes in phase angle and modulation will allow for accurate measurement of pH within EC film. For example, 0.005 pH change in the range from 4.5 to 7.0 will result in phase changes of 0.1 deg and modulation of 0.1 %. The apparent pKa from phase angle (5.76) and modulation (5.82) are shifted slightly compared to the true pKa value of CPR in solution (6.01) determined from the pH dependent absorbance at 572 nm. Interestingly, that pH dependent phase angles and modulation fit well to the well-known Henderson-Hasselboch equation, which allows apply the analytical expression for practical calibrations of FRET-based pH sensors.

In order to measure pH in wide range it would require using several pH indicators with various pKas. The pH sensitive ranges of optical pH sensors are limited usually by their pKa values and are considered as  $\text{pKa} \pm 1.0$ . It was reported that the pH sensitive range can be significantly extended up to 5 pH units using lifetime and wavelength-ratiometric pH probes [9]. In case of FRET-based pH sensors

the sensing pH range usually will be limited to the 2 pH units and in some cases to the 3 pH units i.e.  $pK_a \nabla 1.5$ . In order to extend the pH sensitive range, a combination of two pH indicator CPR and TBPSP were used (Figure 5). In this case the optical pH sensor allow pH measurement in the extended range from pH 5 to almost 10. One may imagine a multiple pH indicators to cover even wider pH range to be measured.

In Figure 6 are shown pH dependent phase angles for several FRET-based pH sensors in the range from pH 1 to pH 11. This has been achieved because the pH sensitive absorption spectra of many pH indicators are overlapping with a Ru(II) emission spectra. Therefore, single Ru(II) complex (e.g. in our case  $[Ru(dpp(SO_3Na_2)_3)]^{2+}$ ) can be used as an energy donor in a number of the FRET-based pH sensors. This approach also allows design the sensors for other analytes that affect the pH value in the sensing element. The most sensitive pH range can be chosen for specific analytes. Using a single fluorescent dye simplify the requirements for one excitation and one observation wavelength (or excitation and observation wavelength bands) as well as for modulation frequency. Additional advantage of using Ru(II) complexes as a donors is almost 200 nm Stokes shifts which allow for very efficient LED excitation (400 -500 nm) and collecting a whole emission spectrum (above 550 nm). Long lifetime of several microseconds allows for low modulation frequencies in the range of several hundred kHz also allow design inexpensive phase-modulation instruments. One can imagine a sensor array for various analytes that performs based on the same principle, FRET from Ru(II) to the various pH indicators, that will be driven by one generic instrument.

#### *Effect of polymeric support on FRET based pH sensor*

Five polymeric supports for pH sensors were examined, including polyHPMA (PHPMA), ethyl cellulose (EC), polyvinyl cellulose (PVC), polyvinyl butyral (PVB), and sol gel (SG). Polymeric effect on the performance of pH sensor is demonstrated using TBPSP as an acceptor (0.75 mM in coating solutions). Ph-dependent phase angles of  $[Ru(dpp(SO_3Na_2)_3)]$ - TBPSP in various polymers were measured using the same buffers. In Figure 7 are shown calibrations for  $[Ru(dpp(SO_3Na_2)_3)]$ - TBPSP in

EC, PHPMA, and PVC using phase angles. The values of  $R_0$  for  $[\text{Ru}(\text{dpp}(\text{SO}_3\text{Na}_2)_3)]^-$  TBPSP estimated based on spectral overlaps indicate that FRET efficiencies are expected comparable in all polymeric matrices (see Table 1). It has been found that depending on polymeric support the apparent  $\text{pK}_a$  constant is shifted towards higher pH values compared to that for free TBPSP in the buffer (7.52). The largest shift has been observed for PVC, 0.95 pH unit and the smallest for PHPMA of 0.16. These data indicate that the pH sensitive range can be tuned by the choice of polymeric support. Also, these data illustrate the differences for TBPSP when in the aqueous buffer and in the polymeric support. The intensity decays of donor have been found the most heterogeneous in Sol-Gel and the least in PHPMA indicating different micro heterogeneity of polymeric matrices.

A number of pH sensitive donor-acceptor systems have been characterized. The FRET between the Ru(II) donor and the pH sensitive acceptors as a transduction mechanism has been used to demonstrate the pH sensing using phase and modulation of donor luminescence. Several pH indicators with different  $\text{pK}_a$  values were chosen to design the pH optical sensors using a Ru(II) complex with a high quantum yield and long lifetime. The wide pH range, from 1 to 11 can be measured with the same optical and electronic rearrangement of the instrumentation.

A series of polymeric supports have been investigated. The results for TBPSP indicate that a donor and acceptor property are affected by the polymeric support. The lifetime of the donor varies from 3.5  $\mu\text{s}$  (EC) to 5.2  $\mu\text{s}$  (PVC) in the absence of oxygen. Observed apparent  $\text{pK}_a$  of pH sensor may be shifted of about 1 pH unit by changing the polymeric supports.

The principles of FRET-based pH sensors can be utilized for design an optical sensors for a variety other analytes that induce the pH changes within a sensing element.

**Table 1.** Fluorescence Energy Transfer Characteristics for Donor  $[\text{Ru}(\text{dpp}(\text{SO}_3\text{Na}_2)_3)]\text{Cl}_2$  and several Acceptors from Phthalein Derivatives (pH indicators).

pH indicator	Polymeric Support	$\tau_D$ ( $\mu$ s)	$R_0$ ( $\Delta$ )		$C_0$ (mM) <sup>a)</sup>
			base	acid	base
Bromothymol Blue	EC	3.48 (1.22) <sup>b)</sup>	43.6	11.7	4.8
Bromophenol Blue	EC	3.48	49.9	32.3	3.2
m-Cresol Purple	EC	3.48	49.5	19.0	3.3
Chlorophenol Red	EC	3.48	49.7	5.5	3.2
TBPSP	EC	3.48	39.6	22.2	6.4
TBPSP	PVC	5.24	42.6	23.1	5.1
TBPSP	PVB	4.70 (3.57)	42.0	23.5	5.4
TBPSP	PHPMA	5.12 (3.31)	37.1	27.8	7.8
TBPSP <sup>c)</sup>	sol gel	3.58 (1.92)	37.3	18.8	7.6
Phenolphthlein <sup>c)</sup>	EC	3.48	40.8	N/A	5.8

<sup>a)</sup>  $C_0$  values were calculated from  $C_0 = 3000/(4\pi NR_0^3)$ , where N is the Avogadro number [24].

<sup>b)</sup> Values in the brackets are in air equilibrated samples.

<sup>c)</sup>  $R_0$  calculated for aqueous solutions.

### Claims

What is claimed is:

1. A method of optically determining concentration of analyte in a sample comprising the steps of:
  - (a) contacting an energy transfer donor-acceptor mixture with a sample to be analyzed, wherein energy transfer between donors and acceptors is affected by the changes of the pH within a sensor medium induced by an analyte in the sample,
  - (b) contacting the sensor with a liquid sample,
  - (c) exciting the sensor with intensity modulated radiation,
  - (d) detecting the resulting emission,
  - (e) performing a calculation consisting essentially of calculation of apparent lifetime of the emission to determine the analyte concentration of the sample.
2. The method of claim 1, wherein the donor is selected from a metal-ligand complexes, such as Ru(II), Os(II) and Re(I),
3. The method of claim 2, wherein the donor is a Tris (disodium 4,7-diphenyl-1,10 phenanthroline sulphonate) ruthenium (II) ( $\text{Ru}[(\text{dpp})(\text{Na}_2\text{SO}_3)]_3 \text{Cl}_2$ ).
4. The method of claim 1, wherein the acceptor is selected from a pH indicators, such as phthaleins.
5. The method of claim 4, wherein the acceptor is selected from the group consisting Bromothymol Blue, Bromophenol Blue, Chlorophenol Red, 3,4,5,6-tetrabromophenolsulfophthalein, m-Cresol Purple, Cresol Red, Phenol Red, and Phenolphthalein
6. The method of claim 1, wherein the donor and acceptor are mixed together in a known ratio,
7. The method of claim 1, wherein the donor is mixed with more than one acceptor in a known ratios,
8. The method of claim 1, wherein the donor and acceptor are bound to a polymeric carrier,
9. The method of claim 8, wherein the carrier is selected from the group consisting EC, PHPMA, PMMA, PVC, PVB, and sol gel.

10. The method of claim 1, wherein the apparent lifetime is calculated using phase-modulation fluorometry or time-resolved fluorometry.
11. The method of claim 10, wherein the calculated phase angle and modulation are used for analyte determination.
12. The method of claim 1, wherein the changes in pH value within a sensing element is induced by an analyte in the liquid samples consisting hydrogen ion, carbon dioxide, sodium, potassium, calcium, magnesium, chloride, ammonia ...



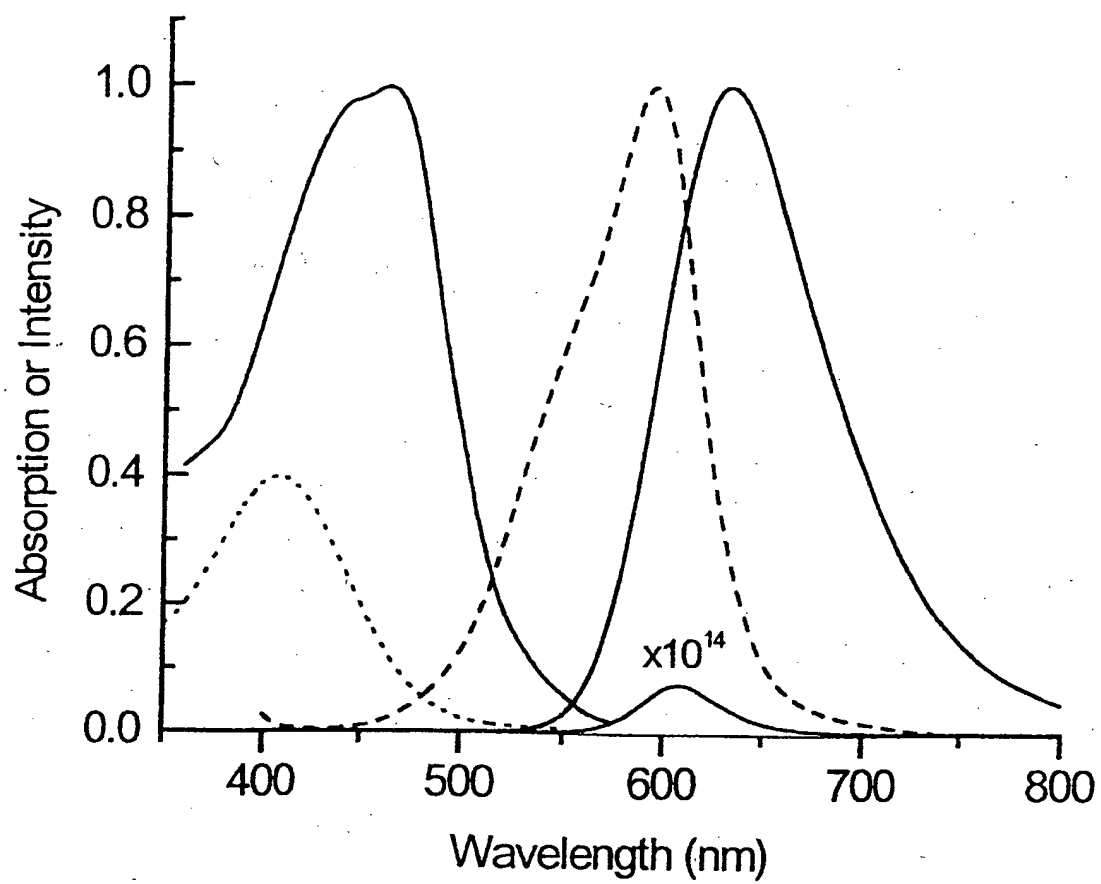


Figure 1

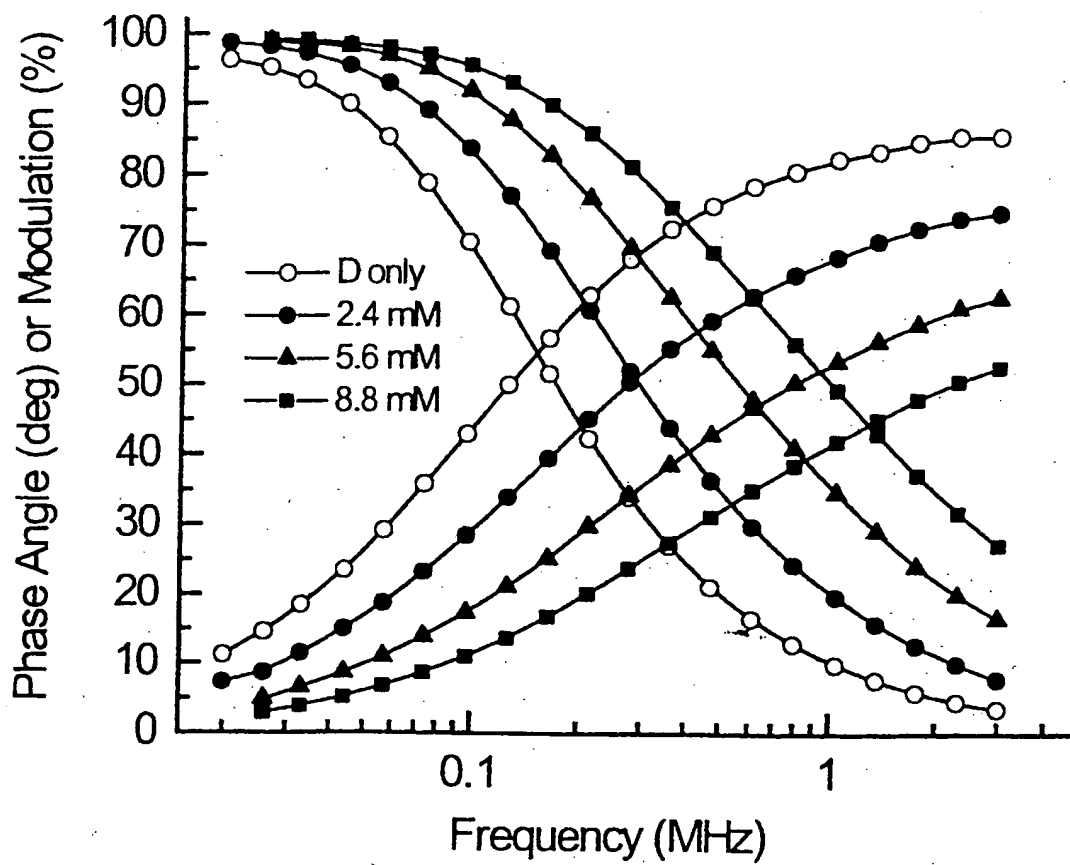


Figure 2

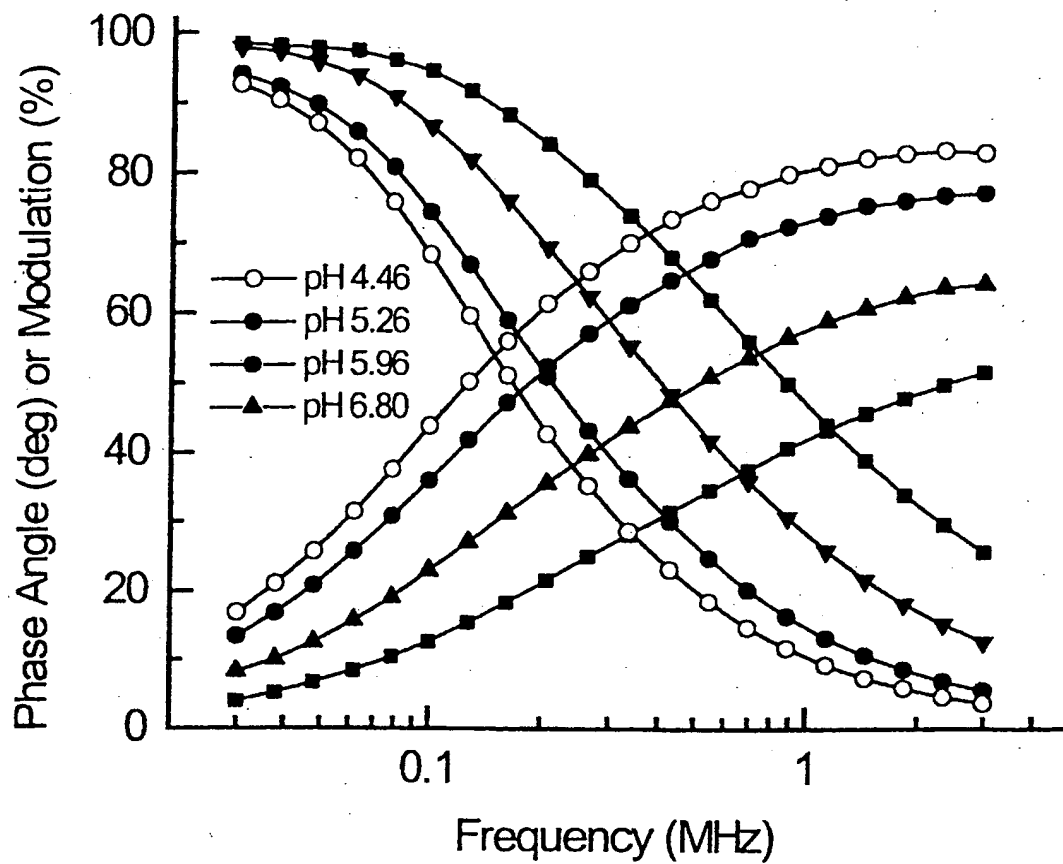


Figure 3

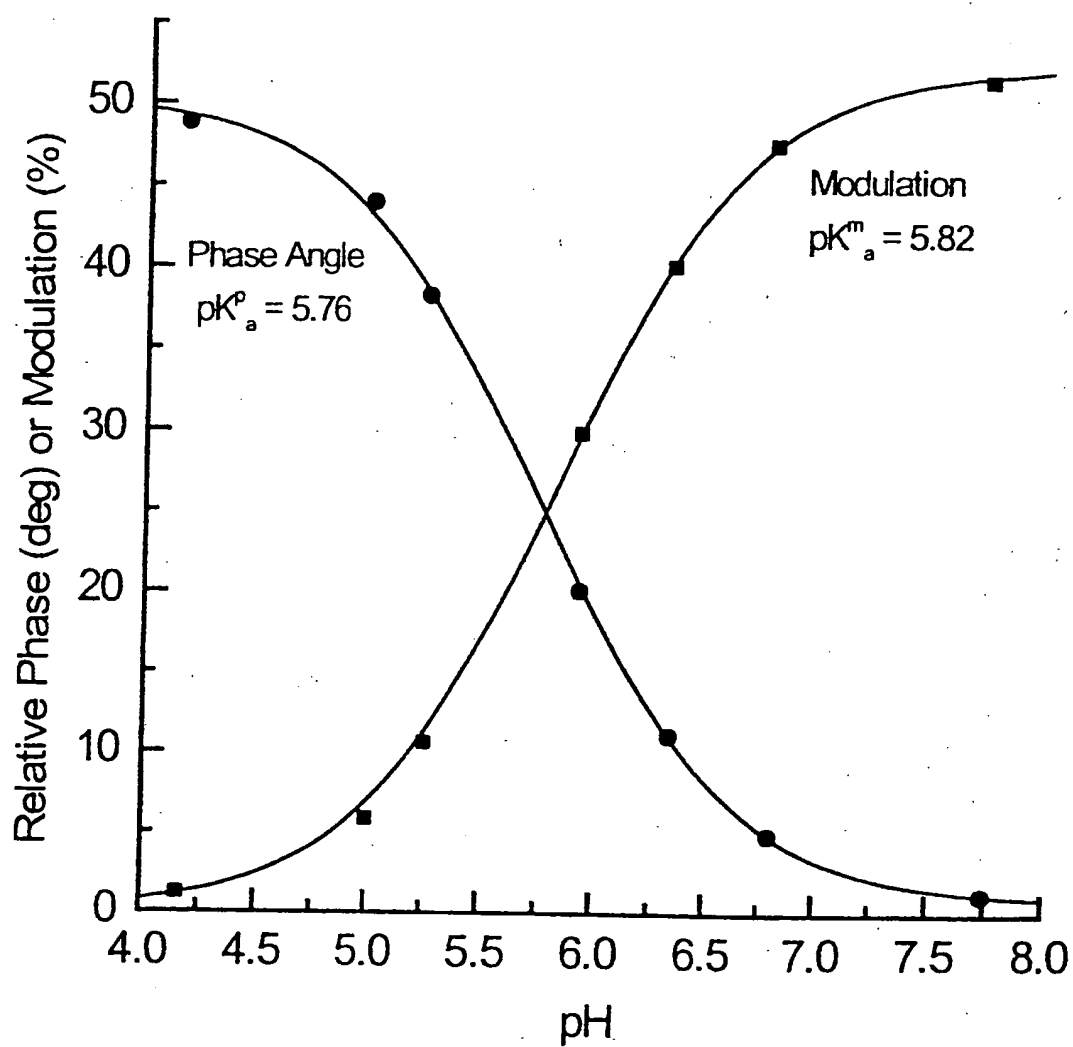


Figure 4

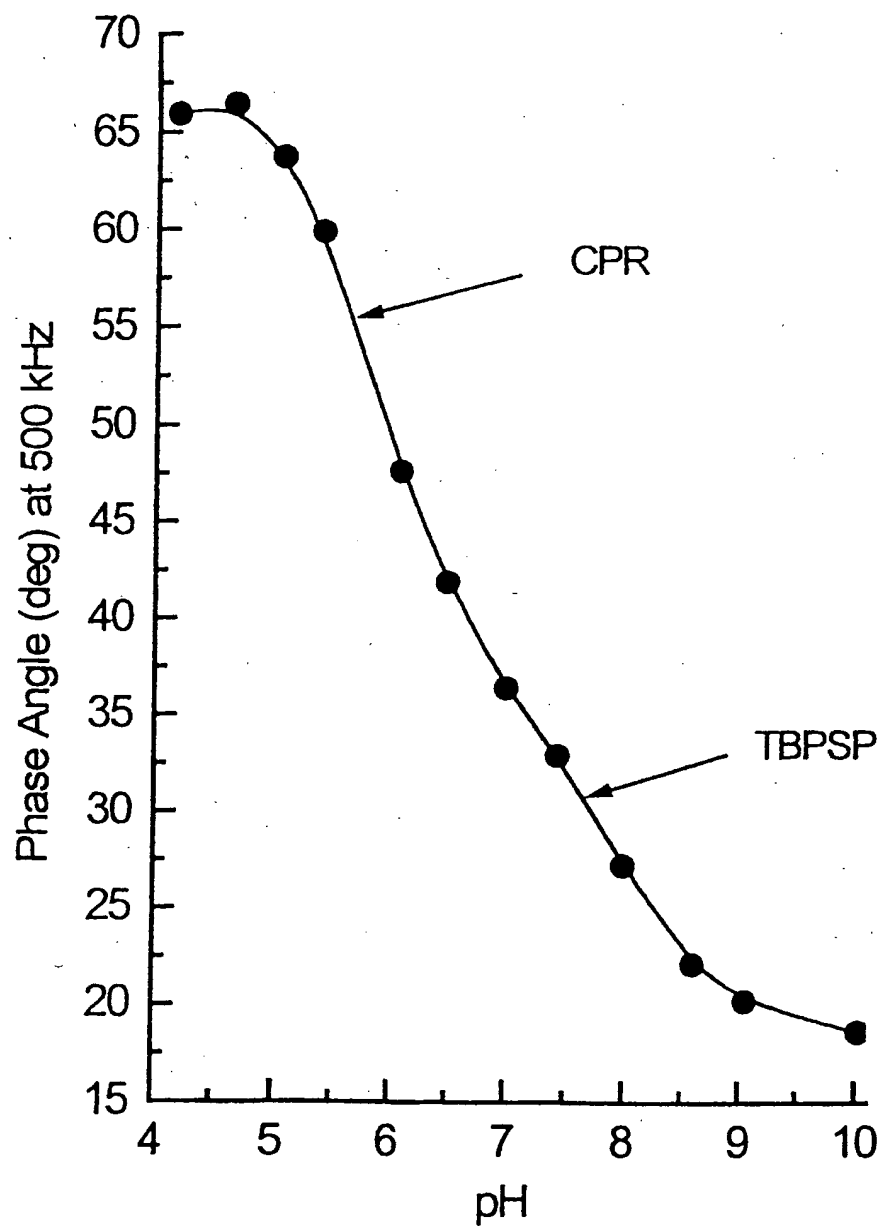


Figure 5

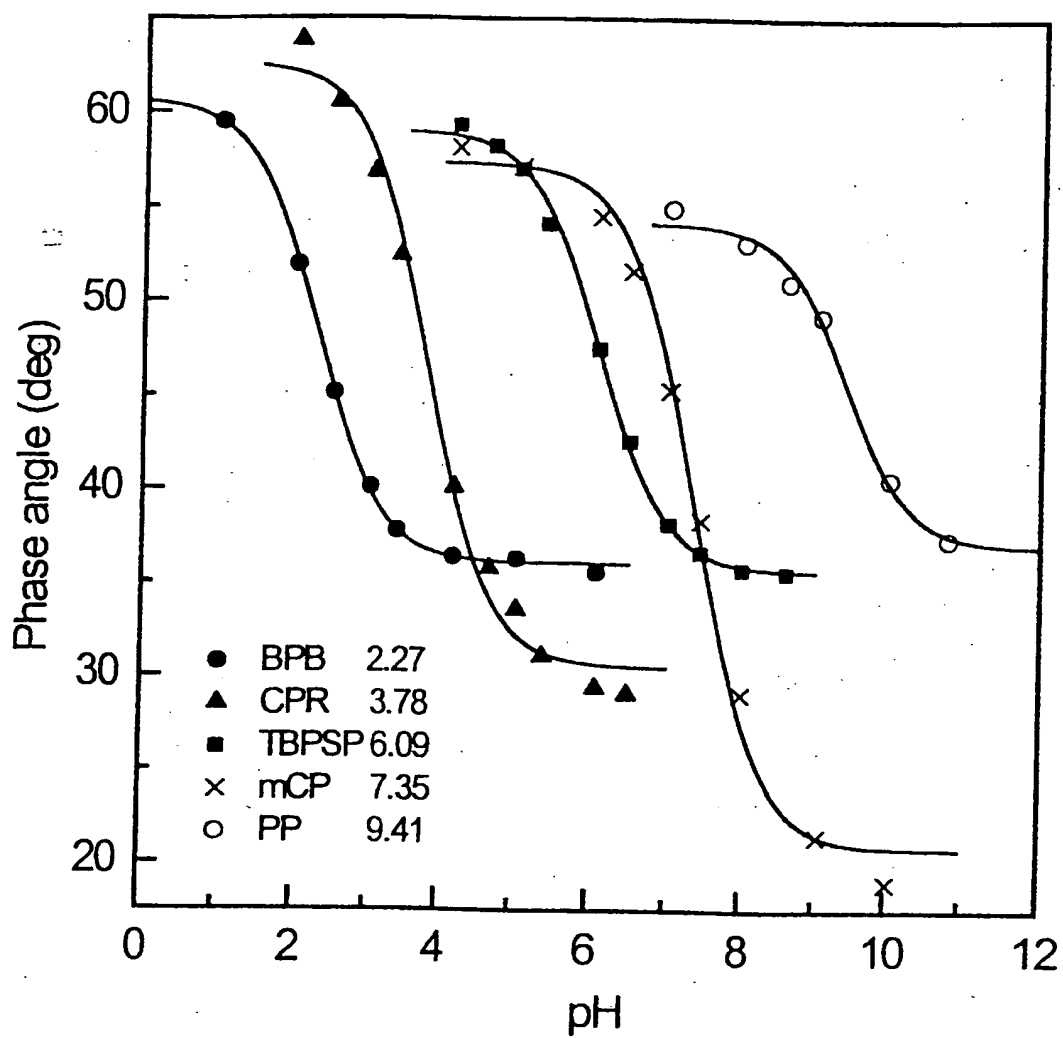


Figure 6

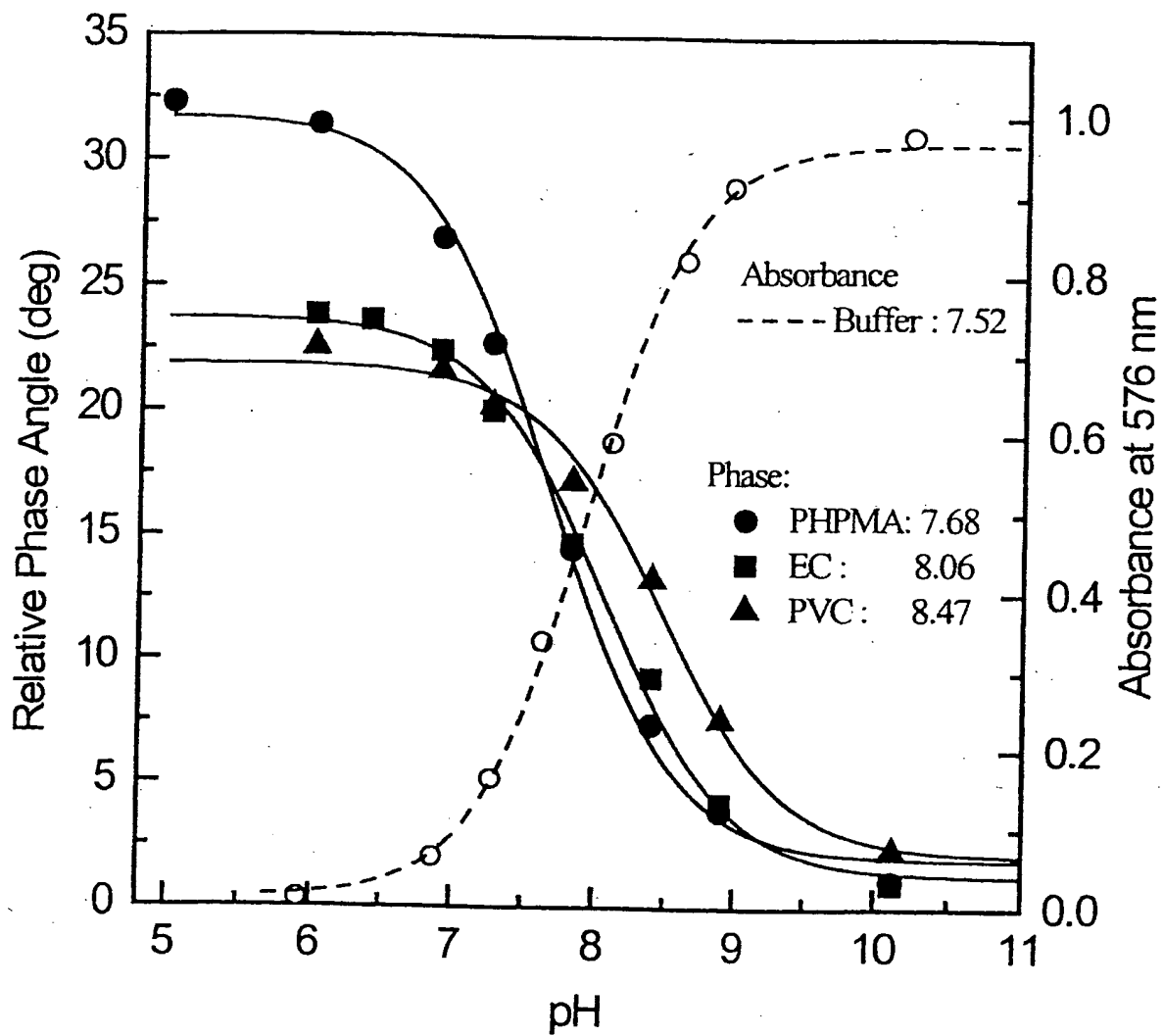


Figure 7

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/14709

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 21/64

US CL : 436/68, 133, 163, 172

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/68, 133, 163, 172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,648,269 A (LAKOWICZ et al) 15 July 1997, claims 1-7 and column 2, line 66 to column 3, line 6.	1-12
Y	US 5,628,310 A (RAO et al) 13 May 1997, entire document.	1-12
A	SIPIOR.J. et al. A Lifetime-Based Optical CO2 Gas Sensor with Blue or Red Excitation and Stokes or Anti-Stokes Detection. Analytical Biochemistry. 1995. Vol. 227. pages 309-318.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

11 AUGUST 1999

Date of mailing of the international search report

10 SEP 1999

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